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Lassa Virus Nucleoprotein Mutants Generated by Reverse Genetics Induce a Robust Type I Interferon Response in Human Dendritic Cells and Macrophages▽

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Lassa virus (LASV; *Arenaviridae*) is responsible for severe hemorrhagic fevers in Africa. LASV nucleoprotein (NP) plays important roles in regulating viral transcription and replication and in inhibiting type I interferon (IFN) production. The NP C-terminal domain contains a 3′-to-5′ exonuclease activity involved in suppressing IFN induction. We have established a murine polymerase (Pol) I reverse genetics system for LASV, showing that residues D389 and G392 of NP were critical for LASV viability, while the D389A/G392A and D389T/392A double mutants were severely altered in the ability to suppress IFN in macrophages and dendritic cells. Assessing their attenuation *in vivo* may open new perspectives in vaccinology.

Lassa fever is a viral hemorrhagic fever caused by an Old World arenavirus, Lassa virus (LASV; family *Arenaviridae*) (45) transmitted by infected *Mastomys natalensis*, a peridomestic rodent (35). It is a major public health concern in regions of endemicity in West Africa and a threat for importation and misuse as a bioterrorism agent in industrial countries (9). The severity of the disease varies from asymptomatic infection to fatal hemorrhagic fever (19, 33, 34). Whether infection leads to death seems to depend on host immune responses, although the mechanisms involved remain to be clarified. LASV tropism for antigen-presenting cells (APC), such as dendritic cells (DC) and macrophages (MP), in the early stages of infection probably plays a key role in the defective cellular responses observed for severe cases (5, 28, 29, 51). DC and MP massively release LASV but are not activated and do not produce cytokines, except for a modest type I interferon (IFN) production (5, 7).

LASV is enveloped and has two single-stranded RNA genome segments (L and S) of ambisense polarity (9). The L segment codes for the small zinc-finger protein Z and for the RNA-dependent RNA polymerase (Pol) L. The S segment codes for the nucleoprotein (NP) and the precursor to the glycoproteins maturated by subtilase SKI-1/S1P (24) into GP1 and GP2. In each segment, the open reading frames (ORFs) are separated by an intergenic region forming a hairpin structure acting as a transcription termination signal for the mRNA synthesis (25, 40, 41, 47). The two RNA segments contribute to virulence. A reassortant exchanging the L segment of LASV with that of the attenuated Mopeia arenavirus (MOPV) is attenuated and protects guinea pigs against a LASV challenge (26, 27). In the S segment, NP plays a role not only in regulating transcription and replication (41) but also in inhibiting type I IFN production by interfering with IFN regulatory factor 3 (IRF-3) activation (32). Reverse genetics systems have been developed for several arenaviruses: lymphocytic choriomeningitis virus (LCMV) (10, 13, 14, 46), Junin virus (1, 13), Pichinde virus (22), and very recently, Lassa virus (2). Single-cycle LCMV (44)- or Junin-based chimeras (3) have been developed as well. Here, we established a reverse genetics system for LASV, enabling us to determine the role of specific amino acid residues of NP in inhibiting IFN production.

We selected the Pol I system, which was successfully used for reverse genetics of segmented negative-strand viruses, such as orthomyxoviruses (36, 37), bunyaviruses (8), and arenaviruses (10, 14). The full-length S and L segments of the AV strain were cloned into pRF108, containing the murine Pol I promoter and terminator (15, 17). The resulting pPolI-S_{AV} and pPolI-L_{AV} plasmids generated S and L antigenomic transcripts, respectively, starting with a nontemplated G required for efficient transcription and replication of arenaviruses (14, 16, 43). While cloning the S segment was relatively straightforward, three steps were required for the L segment, due to strong secondary structures in the intergenic region (IGR), impairing plasmid stability (cloning strategy available upon request). Similar difficulties in the cloning of the Junin virus L segment were described previously (1). We confirmed the published AV strain sequence (18, 48) and added the 19 nucleotides missing at the 3′ and 5′ extremities of the L segment (EMBL accession numbers FR832710 and FR832711). The NP and L coding sequences were cloned into pTM1 plasmids (12) between NcoI and BamHI or StuI, respectively. The recom-

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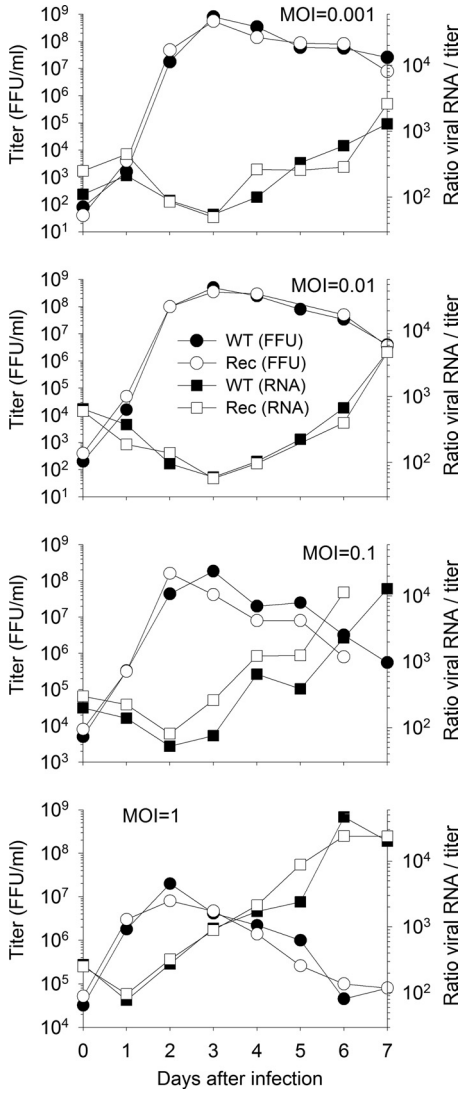

FIG. 1. Comparison of the replication of recombinant versus authentic Lassa virus in Vero E6 cells. Vero E6 cells were infected with WT (black symbols) or Rec-WT (white symbols) LASV at various MOI. In culture supernatants recovered at various time points, viral titers were quantified by titration in Vero E6 (5) and expressed in FFU/ml (circles, left axis), and total RNA from LASV was extracted using TRIzol reagent (Invitrogen) and quantified by real-time reverse transcription-PCR as described in reference 39. The ratio of the number of LASV RNA copies to the infectious titer (square, right axis) was calculated for each supernatant.

binant wild-type (WT) LASV (Rec-WT LASV) was rescued after transfection of pPolI-S_{AV}, pPolI-L_{AV}, pTM1-L, and pTM1-NP (1:1:1:1 ratio) into BHK/T7-9; it could be distinguished from the authentic virus (WT LASV) by synonymous nucleotide changes introduced purposely or by serendipity during the amplification/cloning steps (T1465C and A1486G in the S segment; C2297A, A2459T, and T6762C in the L segment). The yield of Rec-WT LASV in the supernatant increased from 3×10^4 to 3×10^5 focus-forming units (FFU)/ml between days 5 and 6. Viral stocks were produced in Vero E6 cells, and their genetic stability was verified. Rec-WT LASV and WT LASV exhibited similar growth kinetics and produced up to 10^9 FFU/ml (Fig. 1). The yield was reduced significantly in infections at high multiplicities of infection (MOI), suggesting the presence of defective interfering particles (11, 23, 49, 50), as supported by the parallel increase in the viral RNA/infectious particle ratio (Fig. 1).

With the exception of Tacaribe virus, the NP of New and Old World arenaviruses inhibits IFN production and activation of IRF-3 (21, 31, 32, 42). For LCMV, NP residues D382 to R386 (equivalent to D389 to R393 in LASV) bind and interfere with RIG-I/MDA5 activation (30). The crystal structure of the LASV NP showed that the C-terminal domain contains a 3′-to-5′ exonuclease activity, while the N-terminal domain is involved in the cap binding required for transcription (21, 42). Interestingly, the catalytic site of exonuclease activity closely mimics that of the DEDDh family exonucleases with functional residues D389, E391, D466, D533, and H528. *In vitro* assays with NP mutated at these positions have shown that the nuclease activity correlated with the ability to suppress IRF-3 translocation and to block IFN activation. In contrast, the R393A mutation, although proximal to the active site, had no effect on either activity (21, 42).

Combining the data from the LASV NP crystal structure (21, 42) with results for LCMV (30), we used the LASV reverse genetics system to test the role of the critical residues D389 and G392 and the neighboring residue R393 by mutation into alanine in pPol-S_{AV} using QuikChange site-directed (multi)mutagenesis kits (Stratagene) (primers available upon request). Recombinant LASV NP-G392A could not be rescued in BHK/T7-9 cells (three independent trials), suggesting that it is not viable. In contrast, LASV NP-R393A and LASV NP-D389A were rescued. In IFN-deficient Vero E6 cells (Fig. 2), LASV NP-R393A grew almost like the Rec-WT but formed smaller foci. LASV NP-D389A produced a mixed population of large and small foci after two passages with a partial reversion to WT sequence at position 389, indicating that this residue is critical for virus infectivity. Two double mutants, NP-D389A/G392A and NP-D389T/G392A, were also rescued. They appeared genetically stable during at least two passages, probably because of a compensatory effect. However, they formed smaller foci and were more impeded in their replication (30- to 100-fold less yield) than Rec-WT virus (Fig. 2).

To assess the replicative activity of mutated NPs, we established a LASV minigenome system. The virus-like RNA corresponded to the S segment of the AV strain (18), in which the NP ORF (from the AUG codon to the HindIII site) had been replaced by the CAT gene. It was cloned into pRF108, resulting in the pPolI-S_{CAT} plasmid. Transfection of pPolI-S_{CAT}, pTM1-L, and pTM1-NP into BHK/T7-9 cells reconstituted the ribonucleoprotein and allowed minigenome transcription and replication, as monitored by a CAT assay. CAT activity was at its maximum 24 h posttransfection, as for other arenavirus minigenomes (10, 14, 20). A 1:1:1 ratio of pPolI-S_{CAT}/pTM1-L/pTM1-NP was found to be optimal for both WT and mutated NPs, and increasing the amount of NP mostly reduced CAT activity (Fig. 3), as previously observed for arenavirus minigenomes (20, 40). Compared to the CAT activity of WT NP, those of NP-R393A, NP-D389A, and NP-G392A were similar, significantly reduced, and completely reduced, respectively. This strong reduction in minigenome activity of NP-D389A and NP-G392A diverges from pre-

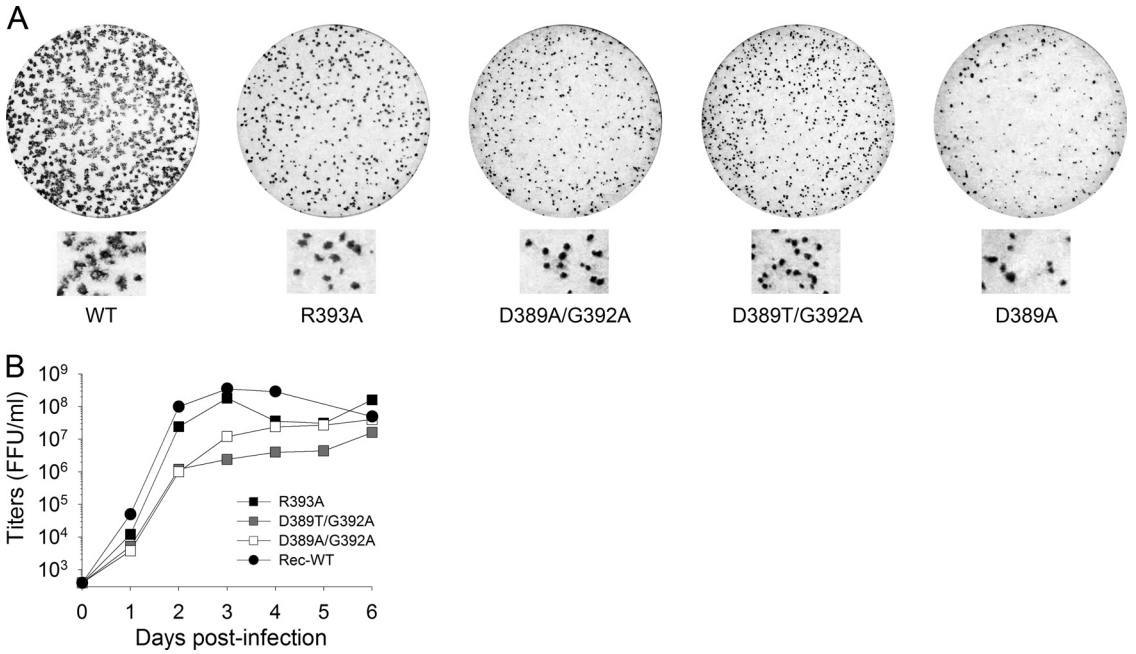

FIG. 2. Replication in Vero E6 cells of recombinant Lassa virus containing mutations into NP. (A) Photographs of foci generated by the different recombinant viruses and revealed by immunostaining. The whole wells and a 3-fold magnification of foci are shown. (B) Vero E6 cells have been infected with mutants NP-R393A (black squares), NP-D389T/G392A (gray squares), or NP-D389A/G392A (white squares) or Rec-WT LASV (black circles) at an MOI of 0.01. Viral titers were determined by titration of culture supernatants in Vero E6 and expressed in FFU/ml.

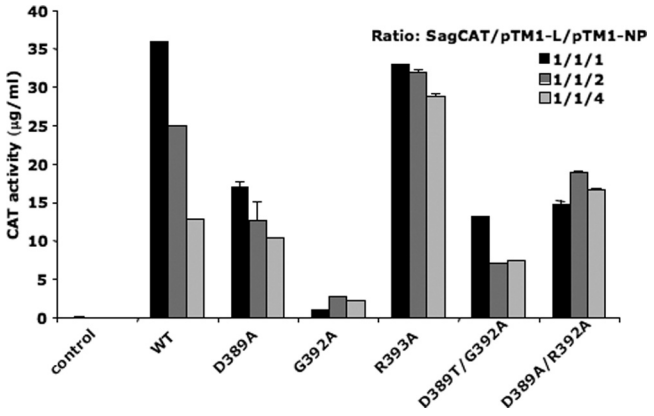

FIG. 3. Replication and transcription activity of mutated NP of Lassa virus in the minigenome system. An NsiI restriction site was introduced by site-directed mutagenesis at the first AUG codon of the NP gene in pPolI-S_{AV}. The CAT gene (660 bp, including a terminal stop codon), flanked by NsiI and HindIII by PCR amplification, was introduced into the modified pPolI-S_{AV} plasmid deleted of the 1,406 nucleotides encompassing the NsiI and HindIII sites. The resulting plasmid was named pPolI-S_{CAT}. CAT activity was assessed by enzyme-linked immunosorbent assay (ELISA) 24 h after transfection of BHK/T7-9 cells (kindly provided by Naoto Ito, Gifu, Japan) using Fugene6 reagent (Roche), with a combination of constant amounts of plasmids expressing the S_{CAT} minigenome (pPolI-S_{CAT}) and the L protein (pTM1-L) and various amounts of plasmid expressing the WT or NP mutants (pTM1-NP) as indicated. The values correspond to CAT activity (μg/ml) measured using a Magellan microplate reader (Bio-Rad) and a quantitative CAT ELISA kit (Roche) according to the manufacturers' instructions.

vious data showing that LASV NP-D389A (and NP-E391A) (42) and the D382A mutation (equivalent to D389 in LASV) in LCMV NP (30) did not affect transcription and replication. For LASV, this difference could be due to the strain variation between AV and Josiah, which differ by 6% in amino acid composition (18). The replicative activity of the double mutants in the minigenome system confirmed that CAT activity was significantly reduced compared to that for WT NP (Fig. 3).

All the LASV mutants, except LASV NP-D389A, were analyzed for their ability to interfere with beta IFN (IFN-β) and IFN-α in human DC and MP cells. LASV, which prevents induction of type I IFN (5) and the closely related MOPV, which is a good inducer (38), were used as controls. As defective interfering (DI) RNAs could induce IFN, we checked RNA copy/FFU ratios in the virus stocks. The double mutants NP-D389A/G392A and NP-D389T/G392A exhibited ratios of 117 and 330, respectively, i.e., about 5- to 10-fold higher than those of the Rec-WT and NP-R393A mutant (24 and 17, respectively). This difference is unlikely to influence type I IFN induction, since WT LASV stocks with ratios of 100 to 300 did not induce type I IFN (4, 6, 7). In DCs and MPs (Fig. 4A and B), Rec-WT and WT LASV replicated similarly and LASV NP-R393A replicated less efficiently (similarly to MOPV), while LASV NP-D389A/G392A and NP-D389T/G392A replicated poorly. To investigate whether this was due to the antiviral effect of IFN, we assessed the mRNA coding for IFN-β, IFN-α1, and IFN-α2 by quantitative real-time PCR (qRT-PCR) in infected cells (Fig. 4C) and the secretion of IFN-α in the culture medium (Fig. 4D). While NP-R393A inhibited IFN activation like Rec-WT and WT LASV, LASV NP-D389A/G392A and NP-D389T/G392A completely lost this inhibitory

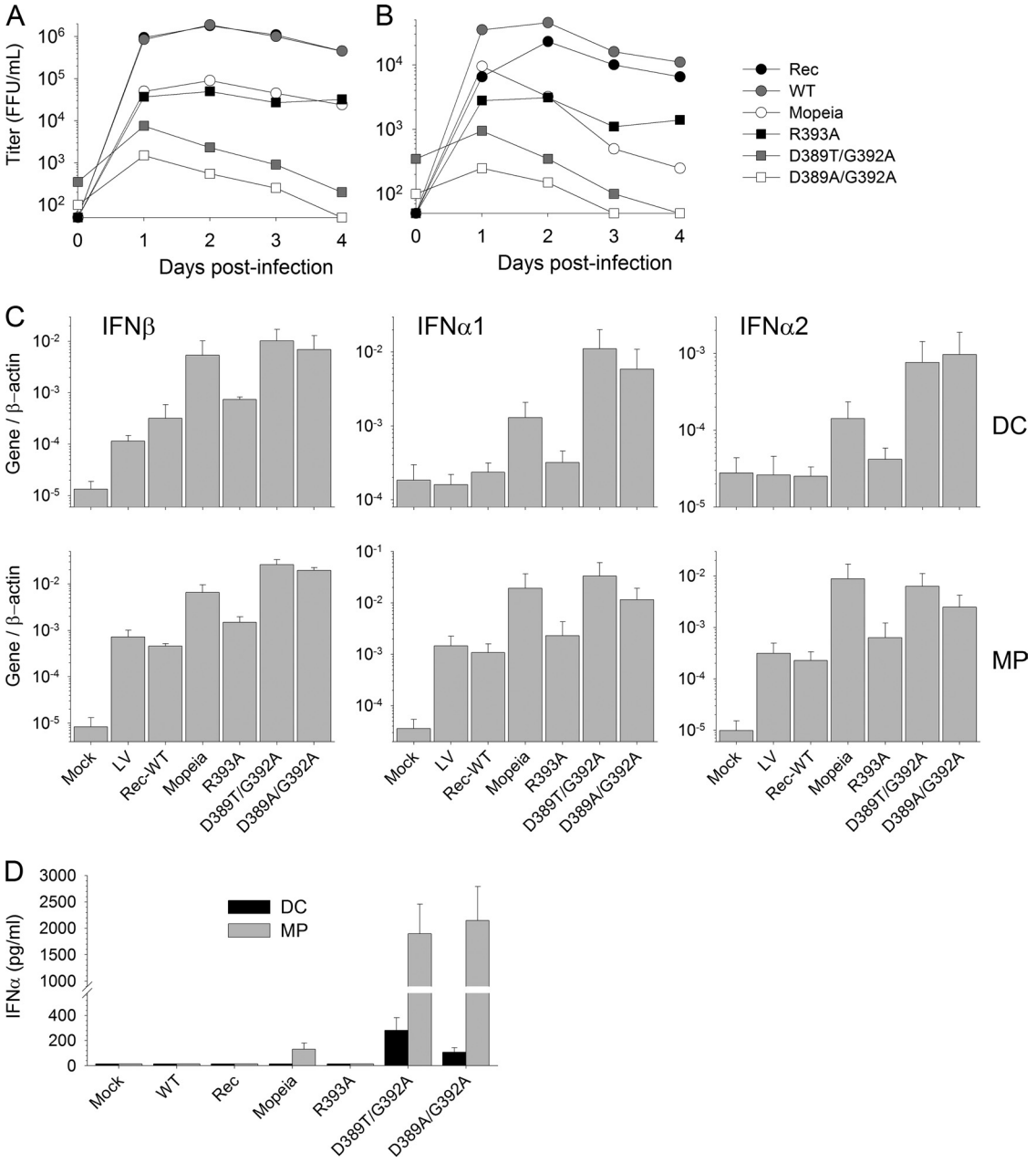

FIG. 4. Replication of authentic and recombinant Lassa viruses in DC and MP and induction of type I IFN. Monocytes were isolated from the blood of healthy donors provided by the Etablissement Français du Sang (Lyon, France) as previously described (5). Human immature monocyte-derived DC (A) or macrophages (B) were infected at an MOI of 0.1 with MOPV (white circles), WT LASV (gray circles) or Rec-WT (black circles), NP-R393A (black squares), or NP-D389T/G392A (gray squares) or NP-D389A/G392A (white squares) LASV. Viral titers were determined by titration of culture supernatants and expressed in FFU/ml. The results presented here correspond to the means from two independent experiments using two blood donors. (C) Total cellular RNA was extracted using TRIzol reagent (Invitrogen). The levels of IFN-β (left graphs), IFN-α1 (middle graphs), and IFN-α2 (right graphs) mRNAs in DC (upper graphs) and MP (lower graphs) mock infected or infected with the different recombinant or WT LASV or MOPV were determined by quantitative RT-PCR 18 to 48 h after infection (MOI = 2) as previously described (39). The results reported are the numbers of copies of the mRNA considered/number of copies of β-actin mRNA and represent the mean ± standard error from three independent experiments (different donors). (D) IFN-α present in supernatants of DC (black bars) or MP (gray bars) 24 to 48 h after infection was assayed with a human-specific ELISA set (Bender MedSystems, Vienna, Austria). The results are expressed in pg/ml and represent the mean ± standard error from three independent experiments (different donors).

effect. They induced IFN mRNAs (Fig. 4C) and IFN secretion (Fig. 4D) at similar or even higher levels than that for MOPV.

In conclusion, NP residue R393 is not crucial for transcription and replication. It does not affect the LASV capacity to counteract IFN induction in primary human DC and MP, although its replication is slightly delayed. In contrast, residues D389 and G392 are critical for LASV viability and replication, but the double mutants D389/G392 were rescued, suggesting a compensatory effect. Replication of these double mutants was severely affected, and their ability to counteract IFN induction in

DC and MP cells was strongly impaired. They produced even more IFN than MOPV, which probably retains some IFN-inhibitory effect, possibly through the DEDDh motif present in its NP protein (30). Investigations of the attenuation of the double mutants *in vivo* will open new perspectives in vaccinology.

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